Please amend claims 1, 9 and 11-14 under the provisions of 37 C.F.R. §1.121(c). The amended claims are presented below and the amendments to the claims are indicated in the marked-up set of claims in Attachment A hereto.

N.E. Clean copy of claims - not. attach.

[PLEASE REFER TO ATTACHMENT A FOR DISCUSSION PURPOSES]

REMARKS

Claims 1-32, 45, 70 and 71 were pending in the subject application, of which claims 20-32, 45, 70 were 71 are withdrawn from consideration. Claims 1-19 are rejected. Applicants have hereinabove canceled claims 4-8, 15-19, 20-32, 45, 70 and 71, and amended claims 1, 9 and 11-14. Accordingly, claims 1-3 and 9-14 are presented for the Examiner's consideration.

Rejection under 35 U.S.C. § 112, first paragraph -Written Description

On pages 2-3 of the March 28, 2001 Office Action, the Examiner rejected claims 1-19 under 35 U.S.C. 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, has possession of the claimed invention. The Examiner cited *In re Rasmussen*, 650 F.2d 1212, 211 U.S.P.Q. 323 (C.C.P.A. 1981), and *In re Wertheim*, 541 F.2d 257, 191 U.S.P.Q. 90 (C.C.P.A. 1976). The Examiner stated that claims 1-19 are essentially drawn toward a method of treating or preventing hepatitis C virus infection in a human subject by administering an effective amount of a DEAD-box protein to the

subject, wherein the DEAD-box protein is capable of specifically binding to the HCV core protein so as to inhibit HCV replication, and a pharmaceutically acceptable carrier is also contemplated. The Examiner stated that the written description requirement under Section 112, first paragraph, sets forth that the claimed subject matter must be supported by an adequate written description that is sufficient to enable anyone skilled in the art to make and use the invention. The examiner stated that the courts have concluded that the specification must demonstrate that the inventor(s) had possession of the claimed invention as of the filing date relied upon. The Examiner stated that although the claimed subject matter need not be described identically, the disclosure relied upon must convey to those skilled in the art that applicants had invented the subject matter claimed, citing *In re Wilder*, et al., 222 U.S.P.Q. 369 (C.A.F.C. 1984), *In re Werthheim*, et al., 191 U.S.P.Q. 90 (C.C.P.A. 1976), *In re Driscoll*, 195 U.S.P.Q. 434 (C.C.P.A. 1977), *Utter v. Hiraga*, 6 U.S.P.Q. 2d 1709 (C.A.F.C. 1988), *University of California v. Eli Lilly*, 119 F.3d 1559, 43 U.S.P.Q. 2d 1398 (Fed Cir. 1997), *Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.*, 18 U.S.P.Q.2d 1016-1301 (C.A.F.C. 1991), *Fiers v. Sugano*, 25 U.S.P.Q.2d 1601-1607 (C.A.F.C. 1993), *In re Bell*, 26 U.S.P.Q. 2d 1529-1532 (C.A.F.C. 1993), and *In re Deuel*, 34 U.S.P.Q. 2d 1210-1216 (C.A.F.C. 1995).

The Examiner alleged that applicants' disclosure fails to provide adequate written support for the invention as claimed. The Examiner alleged that applicants' claims encompass a method of treating or preventing hepatis C virus infection in a human subject by administering an effective amount of a DEAD-box protein to the subject, wherein the DEAD-box protein is capable of specifically binding to the HCV core protein so as to inhibit HCV replication. The Examiner reiterated the allegation that

the disclosure fails to provide an adequate written description for subject matter supporting the *in vivo* DEAD-box inhibition of HCV. The Examiner alleged that applicants' claims are drawn to a treatment based on a hypothesis of inhibition of viral replication which is unsupported by the specification. The Examiner then alleged that the theory that the binding of DEAD-box protein to core protein may actually enhance viral replication may be more likely. The Examiner alleged that mere interaction or binding between HCV core protein and DEAD-box protein in an *in vivo* binding assay is insufficient-especially since a cell culture system is not currently available for HCV. Then, the Examiner reiterated that the binding may actually stimulate viral replication by DEAD-box protein altering viral genomic RNA structure in viral particles in newly infected cells. The Examienr reiterated that the allegation that the consequences of HCV core protein on host cell physiology under natural conditions of infection is difficult to determine since an appropriate cell culture system does not exist.

In response, initially, applicants admit to some confusion as to the basis for the written description rejection. The claims under examination were filed with the application and are reproduced verbatim in the specification. Thus, it is not clear how claims which appear verbatim in the specification can be "not described". According to the M.P.E.P. § 2163.04(I):

> the examiner should: (A) identify the claim limitation not described; and (B) provide reasons why persons skilled in the art at the time the application was filed would not have recognized the **description** of this limitation in the disclosure of the application as filed. A typical reason points out the differences between what is disclosed and what is claimed. A

> simple statement that 'There does not appear to be a written description of the claim limitation '_____' in the application as filed.' may be sufficient where the support is not apparent and the applicant has not pointed out where the limitation is supported. (Emphasis added)

Applicants point out that so long as a "description" of the invention appears in the specification, a "written description" rejection is not appropriate. Whether applicants' description is "enabling" is an issue to be addressed in an enablement rejection, which is what applicants believe the Examiner intended. Accordingly, applicants address any apparent enablement issues in response to the enablement rejection below.

In view of the foregoing, applicants respectfully request that the Examiner reconsider and withdraw the written description rejection.

Rejection under 35 U.S.C. § 112, first paragraph -Enablement

On pages 4-5 of the March 28, 2001 Office Action, the Examiner rejected claims 1-19 under 35 U.S.C. 12, first paragraph, alleging that the specification does not reasonably enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. The Examiner alleged that as set forth above, claims 1-19 are drawn toward a method of treating or preventing hepatitis C virus infection in a human subject by administering an effective amount of a DEAD-box protein to the subject, wherein the DEAD-box protein is capable of specifically binding to the HCV core protein so as to inhibit HCV replication, which is not adequately supported by the disclosure. The Examiner reminded applicants of the legal considerations governing enablement determinations

pertaining to undue experimentation as disclosed in *In re Wands*, 8 U.S.P.Q. 546 (PTO Bd. Pat. App. Int., 1986). The Examiner stated that the courts concluded that several factual inquires should be considered when making such assessments including the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples, the nature of the invention, the state of the prior art, the relative skill of those in that art, the predictability or unpredictability of the art and the breadth of the claims, citing *In re Rainer*, 52 C.C.P.A. 1593, 347 F.2d 574, 146 U.S.P.Q. 218 (1965). The Examiner alleged that the disclosure fails to provide adequate guidance pertaining to these considerations. Moreover, the Examiner alleged that the prior art is unpredictable and fails to provide any guidance pertaining to the functional requirements of the instant method, and the prior art teaches away from the hypothesis which applicants have based their claims upon. Thus, the Examiner alleged that the conclusions drawn in the specification with respect to *in vitro* experiments are not supported by the art. The Examiner alleged that Owsianka and Patel (Virology **257**, 330-340, 1999), when discussing a similar experimental mode, teach that it is important to realize that the model system in which their studies were completed is artificial, and they teach that DDX3 may be sequestered from its normal function by HCV core to cooperate directly in some aspect of the viral life cycle: "If DEAD-box proteins can be thought of as mechanical devices for carrying out various RNA-related processes, then DDX3 could be envisioned as a molecular motor that the virus needs to drive its own machinery for manipulating RNA. It could be involved in the expression, replication, or packaging of viral RNA." Thus, the Examiner alleged that the art is not supportive of applicants' hypothesis that the binding of core protein by DEAD-box protein *in vivo* inhibits HCV viral replication. The Examiner concluded with the

assertion that the disclosure fails to provide sufficient guidance pertaining to the method claimed, thus, when all the aforementioned considerations are taken together, it would clearly require undue experimentation to practice the claimed invention.

In response, applicant respectfully traverse the Examiner's position on the ground that 1) applicants do show *in vivo* data that the DEAD-box protein binds with the HCV core protein, and 2) it was known in the art that the HVC core proteins must form multimers before virion assembly can proceed. It is readily evident to one of skill in the art that if an HCV protein is bound to the DEAD-box protein *in vivo*, the HCV protein cannot multimerize. This, in turn, prevents virion assembly and initiation or progression of the viral infection.

Specifically, the experimental details presented on pages 26-36 of the subject specification are describing an *in vivo* study in the yeast two-hybrid system. The results are presented in Figure 8, for example. Furthermore, Figures 7A and 7B and their corresponding description on pages 9-11 show that HCV core protein and the DEAD box protein bind in mammalian cells as well. Thus, it is clear that the DEAD-box protein binds to the HCV core protein *in vivo*.

It is also known in the prior art that virion assembly requires HCV core protein polymerization. See, for example, the Abstract and the bottom paragraph of page 50, right column, of Matsumoto et al., Virology, 218, 43-51 (1996), copy enclosed as **Exhibit 1.** Because this is known in the prior art, there is no need to have it explicitly stated in the specification. It is well settled that a patent need not teach, and preferably omits, what is well known in the art." M.P.E.P. § 2164.01, citing *In re Buchner*, 929

F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), cert. denied, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984).

Thus, the unavoidable implication of applicants' discovery that the DEAD-box protein binds to the HCV core, *in vivo*, is that the DEAD-box protein prevents the HCV core protein from multimerizing. It is readily apparent and expected that an HCV core protein that has been bound with a DEAD-box protein would not be able to multimerize. According to Matsumoto et al., because the HCV core protein cannot multimerize, virion assembly is hindered. Clearly, if virion assembly is hindered, viral infection is hindered. Thus, applicants' claims are enabled by applicants' discovery taken together with the knowledge in the prior art.

Finally, with respect to the Examiner's citation of Owsianka and Patel, applicants point out that Owsianka and Patel do not deal with the HCV core protein, but only generally with the DEAD-box protein. Furthermore, the quoted portion of Owsianka on Patel is not based on data, but is rather speculative commentary. Applicants' *in vivo* data is, therefore, more relevant to HCV then the comments of Owsianka and Patel.

Accordingly, applicants respectfully request that the Examiner reconsider and withdraw the enablement rejection of the amended claims.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number

provided below.

No fee, other than the enclosed $445.00 extension of time fee, is deemed necessary in connection with the filing of this Response. However, if any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

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I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

Gary J. Gershik 9/28/01
John P. White Date
Reg. No. 28,678
Gary J. Gershik
Reg. No. 39,992

Attachment A
Claims with Amendments Shown

1. A method of treating or preventing hepatitis C virus infection in a subject which comprises administering to the subject an effective amount of ~~an agent to the subject, wherein the agent is~~ a DEAD-box protein capable of specifically binding to the HCV core protein so as to inhibit hepatitis C virus replication.

2. The method of claim 1, wherein the hepatitis C virus infects the liver of the subject.

3. The method of claim 1, wherein the hepatitis C virus infects the liver of a human.

~~4. The method of claim 1, wherein the agent is capable of specifically binding to the HCV core protein having an amino acid sequence of Figure 2, SEQ ID NO:1.~~

~~5. The method of claim 1, wherein the agent binds to the cytoplasmic domain of HCV core protein which comprises amino acid residues 1-123 of said HCV core protein of Figure 2, SEQ ID NO:1.~~

~~6. The method of claim 1, wherein the agent is a polypeptide, a pseudo enzyme, a peptidomimetic compound, a nucleic acid molecule, an antibody or variant thereof.~~

~~7. The method of claim 1, wherein the agent comprises a cellular protein.~~

~~8. The method of claim 7, wherein the cellular protein comprises a DEAD-box protein, or a 14-3-3 protein.~~

9. The method of claim ~~8~~ 1, wherein the DEAD-box protein comprises a DEAD-box RNA helicase.

10. The method of claim 9, wherein the DEAD-box RNA helicase comprises a human DEAD-box protein DBX or a variant thereof.

11. The method of claim 10, wherein the human DEAD-box protein DBX comprises amino acids having the amino acid sequence ~~of Figure 2,~~ SEQ ID NO:1.

12. The method of claim 10, wherein the variant of the human DEAD-box protein DBX comprises amino acids having the amino acid sequence ~~of Figure 2,~~ SEQ ID NO:2.

13. The method of claim 10, wherein the variant of the human DEAD-box protein DBX comprises amino acids having the amino acid sequence ~~of Figure 3,~~ SEQ ID NO:3.

14. The method of claim 10, wherein the variant of the human DEAD-box protein DBX comprises 100-200 amino acid residues ~~which mimics~~ of the amino acid sequence ~~of Figure 2,~~ SEQ ID NO:1 or the amino acid sequence ~~of Figure 3,~~ SEQ ID NO:3.

~~15. The method of claim 8, wherein the 14-3-3 protein comprises the amino acid sequence of Figure 4, SEQ ID NO:5 or a variant thereof.~~

~~16. The method of claim 15, wherein the variant of said 14-3-3 protein comprises 50-200 amino acid residues which mimics~~

~~the active site of said 14-3-3 protein of Figure 4, SEQ ID NO:5.~~

~~17. The method of claim 1, wherein the agent comprises nucleic acid molecule encoding DEAD-box protein of Figure 2, SEQ ID NO:1 or a variant thereof.~~

~~18. The method of claim 1, wherein the agent comprises nucleic acid molecule encoding 14-3-3 protein of Figure 4, SEQ ID NO:5 or a variant thereof.~~

~~19. The method of claim 1, wherein the agent is administered with a pharmaceutically acceptable carrier.~~

VIROLOGY 218, 43–51 (1996)
ARTICLE NO. 0164

Homotypic Interaction and Multimerization of Hepatitis C Virus Core Protein

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Hepatitis C virus (HCV) core protein constitutes a viral nucleocapsid and may possess multiple functions. In this study, we demonstrated the homotypic interaction and multimerization of HCV core protein *in vitro* and *in vivo*. By using a yeast two-hybrid system, we showed that the amino-terminal hydrophilic portion (amino acids 1–115) of the core protein could interact with itself. Deletion analysis mapped the interacting domain within amino acid residues 36–91. The homotypic interaction of the core protein was also confirmed by *in vitro* protein–protein blotting assay using the recombinant HCV core proteins and by its binding to the glutathione *S*-transferase core fusion protein. The biological significance of the core protein self-interactions was demonstrated by the detection of multimeric forms of the core protein in mammalian cells. The domain responsible for multimerization was determined to be within the amino-terminal hydrophilic region (amino acids 1–115). Both the membrane-bound and the free core proteins exist in dimeric and multimeric forms, suggesting that multimerization of the HCV core protein occurred at an early stage of viral assembly and that the multimer forms may be involved in multiple functions of the core protein.

INTRODUCTION

Hepatitis C virus (HCV), a member of the Flaviviridae family, is a positive-stranded RNA virus (Choo *et al.*, 1991; Kato *et al.*, 1990; Takamizawa *et al.*, 1991) which usually causes persistent infection, resulting in chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Aach *et al.*, 1991; Alter *et al.*, 1992; Di Bisceglie *et al.*, 1991; Kiyosawa *et al.*, 1990; Tassopoulos *et al.*, 1992; Tremolada *et al.*, 1991). The viral structural and nonstructural proteins are produced by cleavage of a large polyprotein precursor by both host cell signal peptidases and viral proteinases (Hijikata *et al.*, 1991; Grakoui *et al.*, 1993). Among the viral structural proteins, core protein, which is derived from the amino terminus of the polyprotein, likely forms the nucleocapsid of the virion because it is highly basic and similar in many biological properties to the nucleocapsid proteins found in other flaviviruses (Grakoui *et al.*, 1993; Harada *et al.*, 1991; Selby *et al.*, 1993). The nucleotide and predicted amino acid sequences of the core protein genes are well conserved among different HCV isolates (Bukh *et al.*, 1994). Recent studies have demonstrated that HCV core protein bound to cell membranes both *in vitro* and *in vivo* and also bound the 60S ribosomal subunit and RNA *in vitro* (Santolini *et al.*, 1994). The ribosome- and RNA-binding domains were mapped to the amino-terminal region between amino acid residues 1 and 75 of the core protein (Santolini *et al.*, 1994). HCV core protein has also been shown to suppress the replication and gene expression of hepatitis B virus (HBV) (Shih *et al.*, 1993). In addition, it has been shown to be phosphorylated by protein kinase A and protein kinase C, and phosphorylation is essential for the HCV core protein to suppress HBV replication and gene expression (Shih *et al.*, 1995). The phosphorylation sites were mapped to Ser-99 and Ser-116 but not Ser-53 (Shih *et al.*, 1995). Finally, HCV core protein activated human c-*myc*, Rous sarcoma virus long terminal repeat (LTR), and simian virus 40 (SV40) early promoters and suppressed the c-*fos* promoter and human immunodeficiency virus type 1 LTR activity (Ray *et al.*, 1995). Taken together, these results suggest that the HCV core protein is a multifunctional protein.

Nucleocapsid formation of virion particles probably involves multimerization of core protein and its interaction with viral RNA. The RNA-binding properties of HCV core protein have been reported (Santolini *et al.*, 1994; Hwang *et al.*, 1995). However, the possible multimerization properties have not been examined. Recent studies have shown that core proteins of many different viruses can oligomerize. For example, the gag proteins of the human immunodeficiency virus, which include matrix, capsid, and nucleocapsid proteins, have been shown by a yeast two-hybrid system to form homodimers; the sequence necessary for the multimerization was located at the carboxy termini of the nucleocapsid and capsid proteins

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0042-6822/96 $18.00

Luban *et al.*, 1992; Franke *et al.*, 1994). The core protein of HBV also forms a multimer via its carboxy-terminal sequence; the truncation of this sequence affects capsid formation and encapsidation of HBV pregenomic RNA (Seames and Lanford, 1993; Birnbaum and Nassal, 1990; Zhou and Standring, 1992; Chang *et al.*, 1994). Here we report homotypic interaction and multimerization of HCV core protein *in vitro* and *in vivo*. This homotypic interaction may be important for HCV assembly and other biological activities.

MATERIALS AND METHODS

Plasmid constructions

The HCV cDNA fragment representing the entire core protein-coding region (amino acids 1–191) of the HCV-T strain (Chen *et al.*, 1992) was generated by polymerase chain reaction (PCR) using two primers corresponding to the 5'- and 3'-ends of the core protein gene plus *Bam*HI sites and was subcloned into the unique *Bam*HI site of the yeast plasmids pGBT9 and pGADGH (Clontech, Palo Alto, CA). The resulting plasmid pGBT9/Core(1-191) and plasmid pGADGH/Core(1-191) encode the GAL4 DNA-binding domain fused to the full-length HCV core protein and a GAL4 activation domain–HCV core fusion protein, respectively. Deletion mutants of HCV core proteins were also generated by similar methods using specific primers corresponding to different regions of the core genes. The resulting plasmids, pGBT9/Core(1-115), pGBT9/Core(1-91), pGBT9/Core(1-26), pGBT9/Core(36-115), and pGBT9/Core(36-91), encode a GAL4 DNA-binding domain fused to the different regions of HCV core protein. pGADGH/Core(1-115) encodes a GAL4 activation domain fused to amino acids 1–115 of the core protein.

To construct the plasmid expressing HCV core protein in *Escherichia coli*, a PCR-generated *Bam*HI fragment containing the full-length core gene of HCV-T strain was cloned into the *Bam*HI site of pGEX-4T-1 (Pharmacia, Uppsala, Sweden). The resulting plasmid, pGEX-HCV-core, encodes a full-length core protein fused to a glutathione S-transferase (GST) protein.

For construction of the plasmid used for *in vitro* transcription, a *Bam*HI fragment containing the full-length core sequence of the HCV-T strain was cloned into the *Bam*HI site of plasmid pCDNA3 (Invitrogen, San Diego, CA). The resulting plasmid, pCDNA3/core, encodes the full-length HCV core protein (a.a. 1–191) under the cytomegalovirus (CMV) immediate-early promoter and T7 promoter.

For expression of HCV core protein in mammalian cells, vector pCMV, which is a derivative of pCDNA3, was used. To construct pCMV, a *Nru*I–*Hin*dIII fragment was removed from pCDNA3 and replaced with the *Eco*RI–*Not*I fragment of pCMVβ (Clontech), resulting in the removal of the T7 promoter and the insertion of the SV40 splicing donor and acceptor sequence into the region between the CMV promoter and the multiple cloning sites of pCDNA3. The full-length core gene of the HCV-T sequence was cloned into the *Eco*RV site of pCMV, yielding plasmid pCMV/core(1-191), which contains the HCV core gene sequence under the control of the CMV immediate-early promoter. Plasmids expressing the truncation mutants of HCV core protein, pCMV/core(1-153) and pCMV/core(1-115), were generated by the same method using different PCR fragments representing the desired sequences.

Yeast two-hybrid system

The *Saccharomyces cerevisiae* strain PCY2 (Chevray and Nathans, 1992) was grown in YPD (1% yeast extract, 2% peptone, 2% dextrose) or synthetic minimal medium (0.67% yeast nitrogen base, 2% dextrose with appropriate auxotrophic supplements). Yeast was cotransformed with GAL4 DNA-binding-domain plasmids and GAL4 activation plasmids by the lithium acetate method (Gietz *et al.*, 1992) and selected for leucine and tryptophan prototrophy. β-Galactosidase activity was assayed on nitrocellulose filter replicas of yeast transformants. Filters were placed in liquid nitrogen for 30 sec and incubated for 8 hr in buffer containing 4 m*M* 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). Positive interactions were indicated by the appearance of blue colonies.

GST fusion protein binding assay

Plasmid pGEX-HCV-core, which expresses GST–HCV core fusion protein, was grown in *E. coli* strain BL21(DE3) (Novagen, Madison, WI) and induced with 1 m*M* isopropylthio-β-D-galactoside (IPTG). Bacteria were pelleted 3 hr after IPTG induction, washed with phosphate-buffered saline (PBS), and resuspended in lysis buffer (1% Triton X-100 in PBS). The suspended bacteria were sonicated on ice for 40 sec and pelleted by brief centrifugation. Supernatants were incubated with glutathione–Sepharose 4B beads (Pharmacia) overnight at 4°. The beads were collected by brief centrifugation in a microfuge and washed four times in 1% Triton X-100 in PBS and once in PBS. Plasmid pCDNA3/core was transcribed *in vitro* using T7 RNA polymerase, and the resulting RNA was translated in a rabbit reticulocyte lysate with [^{35}S]-methionine. Approximately equal amounts, as judged by Coomassie blue staining, of GST or GST–HCV core fusion protein on glutathione–Sepharose beads were incubated with the [^{35}S]methionine-labeled proteins in incubation buffer [40 m*M* HEPES (pH 7.5), 100 m*M* KCl, 0.1% Nonidet P-40, and 20 m*M* 2-mercaptoethanol] for 2 hr at 4° and then rinsed four times in incubation buffer. The beads were boiled in Laemmli sample buffer (Laemmli, 1970), and the supernatants were analyzed by electrophoresis on 15% polyacrylamide gel containing SDS. Afterwards, the gel was dried and autoradiographed.

Far-Western protein–protein blotting

Recombinant HCV core protein expressed in *E. coli* (Lo *et al.*, 1995) and hepatitis delta antigen (of hepatitis delta virus) expressed by recombinant baculovirus (Hwang *et al.*, 1992) were lysed by Laemmli sample buffer, separated by SDS–PAGE on a 15% polyacrylamide gel, and stained with Coomassie brilliant blue or electrotransferred to a nitrocellulose membrane. The membrane was washed with buffer A [10 m*M* HEPES–KOH (pH 7.5), 60 m*M* KCl, 1 m*M* EDTA and 1 m*M* 2-mercaptoethanol] and incubated with 6 *M* guanidine HCl for 15 min at 4° and then sequentially with 3 *M*, 1.5 *M*, 0.75 *M*, 0.38 *M*, 0.19 *M*, and 0.09 *M* guanidine HCl for 5 min each to renature the proteins. The membrane was subsequently blocked for 1 hr at 4° with 5% nonfat dry milk in buffer A containing 0.05% Nonidet P-40. The *in vitro* translated, [^{35}S]methionine-labeled HCV core protein was incubated with the membrane in buffer A containing 3% nonfat dry milk and 0.05% Nonidet P-40 overnight at 4°. Unbound proteins were removed by washing three times with buffer A containing 1% nonfat dry milk and 0.05% Nonidet P-40. Protein binding was detected by autoradiography.

DNA transfections

COS 7 cells were used for DNA transfection. The cells were seeded in 10-cm dishes and cultured at 37° under 5% CO_2 for 24 hr in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. After medium changes, cells were transfected with 20 μg plasmid DNA by the calcium phosphate precipitation method (Chen and Okayama, 1987). The medium was changed after 14 hr of incubation, and cells were collected 48 hr later. Cells were washed with ice-cold PBS twice and harvested.

Membrane flotation analysis

The membrane flotation method was performed as described by Sanderson *et al.* (1993). Cells transfected with DNA were suspended in 0.5 ml hypotonic lysis buffer (10 m*M* Tris HCl, pH 7.5, 10 m*M* KCl, 5 m*M* $MgCl_2$) and incubated on ice for 10 min before disruption of the cells by passage through a 26-gauge hypodermic needle 15 times. Unbroken cells and nuclei were removed by centrifugation at 1000 *g* for 5 min, and the resulting supernatant was subjected to fractionation by the membrane flotation method as described (Sanderson *et al.*, 1993). Briefly, 0.5-ml aliquots of lysates were dispersed into 2 ml of 72% (wt/wt) sucrose in low-salt buffer (LSB) (60 m*M* Tris–HCl, pH 7.5, 25 m*M* KCl, 5 m*M* $MgCl_2$) and overlaid with 2.5 ml of 65% (wt/wt) sucrose in LSB and 0.6 ml of 10% (wt/wt) sucrose in LSB. Sucrose gradients were then centrifuged in a Beckman SW55Ti rotor at 4° for 12 hr at 38,000 rpm. After centrifugation, 0.8-ml fractions were collected successively from the top of the gradient. Any material pelleted by the centrifugation was resuspended in 0.8 ml LSB and designated as the final fraction of the gradient. All fractions were diluted with 4 ml of LSB and recentrifuged in a Beckman SW65Ti rotor at 46,000 rpm for 90 min at 4°, and the resulting pellets were dissolved in LSB.

Cross-linking of proteins by glutaraldehyde

Proteins in cellular lysates or collected from sucrose gradients were incubated with 0.01% glutaraldehyde (Sigma, St. Louis, MO) in LSB for various time periods at room temperature. The reaction was stopped by addition of an equal volume of 2× Laemmli sample buffer (Laemmli, 1970). After heating at 80° for 10 min, the cross-linked proteins were separated by SDS–PAGE and electrotransferred to nitrocellulose membrane for immunoblot analysis.

Immunoblot detection of HCV core protein

The proteins blotted onto nitrocellulose membrane were first treated with 5% nonfat milk in PBS for 60 min and then incubated with rabbit polyclonal anti-HCV core antibody (diluted 1:500) for 2 hr at room temperature. After three washes in PBS, the blots were incubated with ^{125}I-labeled protein A for 2 hr at room temperature. The membrane was washed, and bound antibody was detected by autoradiography.

RESULTS

Detection of the homotypic interaction of HCV core protein in the yeast two-hybrid system

To determine whether HCV core protein can undergo homotypic interaction, we first employed the yeast two-hybrid system (Fields and Song, 1989; Chien *et al.*, 1991). For this purpose, HCV core protein was fused to the C termini of the DNA-binding domain or the activation domain of GAL4 in yeast plasmids pGBT9 and pGADGH, respectively. We first tested four combinations of plasmids, which encoded different lengths of HCV core protein [either full-length (a.a. 1–191) or hydrophilic domain only (a.a. 1–115)] (Fig. 1). The appearance of blue colonies was scored as a positive reaction. The results showed that the full-length HCV core protein (a.a. 1–191) fused with either the activation or the DNA-binding domain of GAL4 did not yield any positive colonies. However, when both the activation domain and the DNA-binding domain contained a partial HCV core sequence from a.a. 1–115, which represents the hydrophilic portion of the core protein, positive colonies were detected (Table 1). Control plasmids expressing only a partial GAL4 protein without the HCV core sequence did not induce β-galactosidase. This result suggests that HCV core proteins can interact with each other through their hydrophilic domains, amino acids 1–115. The failure of the

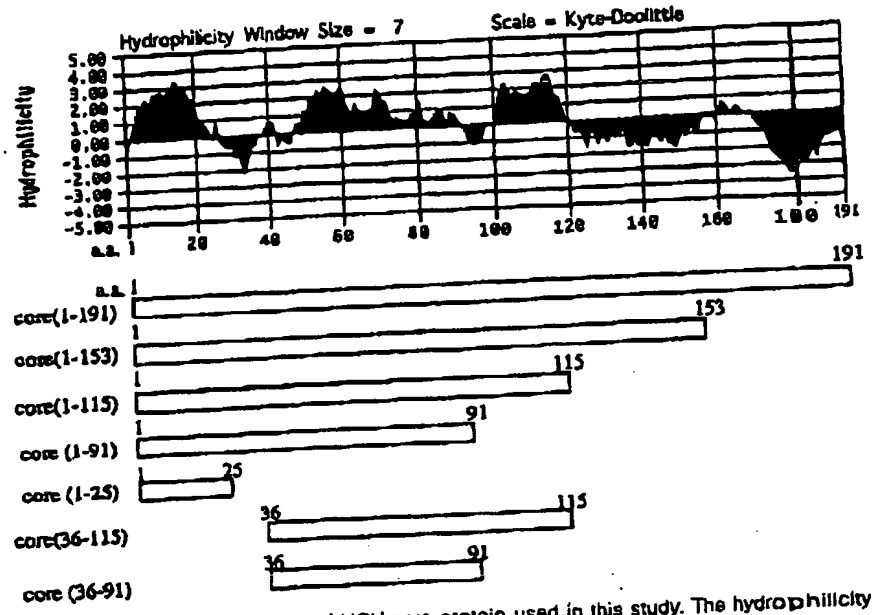

FIG. 1. Schematic representation of the deletion mutants of HCV core protein used in this study. The hydrophilicity plot of the core protein was derived using the MacVector program (IBI, New Haven, CT). The positions of the amino acid residues encoded by each construct are indicated above the boxes.

full- ength HCV core proteins to interact in the yeast two-hybrid system was probably due to the presence of the hydrophobic domain, which may interfere with the nuclear transport of the fusion proteins in the yeast, thus invalidating this assay.

Mapping of the protein-interacting domain of HCV core protein

To further define the domain of the HCV core protein responsible for its homotypic interactions, we constructed several plasmids encoding a GAL4 DNA-binding domain fused with various truncated core proteins (Fig. 1). The resulting plasmids were cotransformed with pGADGH/core(1-115) into yeast and assayed for β-gal induction. Results showed that the truncated core proteins containing either a.a. 1–91 or a.a. 36–115, but not a.a. 1–25, could interact with core a.a. 1–115 (Table 1), suggesting that the homotypic interacting domain of the core proteins is located within a.a. 36–91. However, a.a. 36–91 induced only a weak, though reproducible, interaction (Table 1), suggesting that neighboring regions of the core protein around a.a. 36–91 also contribute to the homotypic interaction of HCV core protein.

TABLE 1

Homotypic Interaction of HCV Core Protein Detected by the Yeast Two-Hybrid System

	β-Galactosidase induction[a]		
	Activation domain hybrid		
DNA-binding domain hybrid	a.a. 1–191	a.a. 1–115	Vector alone
a.a. 1–191	0/33	0/110	0/80
a.a. 1–115	0/56	11/14	0/64
a.a. 1–91	n.t[b]	38/52	0/71
a.a. 36–115	n.t	14/50	0/105
a.a. 1–25	n.t	0/37	0/56
a.a. 36–91	n.t	3/110	0/95
Vector alone	n.t	0/118	0/86

[a] No. of positive/no. of scored clones.
[b] n.t., not tested.

Homotypic interaction of HCV core protein *in vitro*

Since the homotypic interaction of core proteins detected in the yeast two-hybrid system could have been mediated by a yeast protein, we performed *in vitro* biochemical binding assays to determine whether the HCV core protein can self-interact directly. We constructed a plasmid, pGEX-HCV-core, which expressed a full-length core protein fused to GST protein in *E. coli*. The GST–HCV core fusion protein was bound to glutathione–Sepharose 4B beads and incubated with the *in vitro* translated, [^{35}S]methionine-labeled HCV core protein. The bound protein was analyzed by SDS–PAGE. Figure 2 shows that ^{35}S-labeled HCV core bound GST–HCV core fusion protein but not GST. This result indicated that HCV core protein can interact with itself directly. It is interesting to note that while the full-length core protein did not induce β-galactosidase activity in the yeast two-hybrid system, the GST fusion protein containing the full-length core protein did interact with itself in the direct binding assay. Thus, the hydrophobic sequence of the

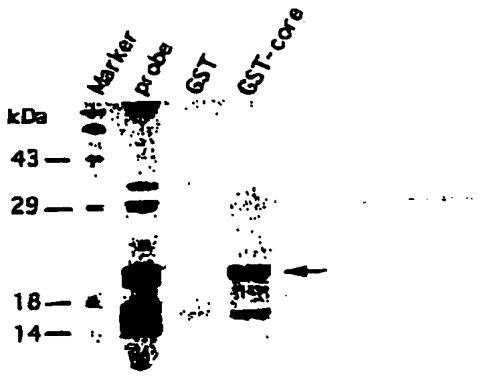

FIG. 2. Homotypic interaction of HCV core protein *in vitro*. Approximately equal amounts of GST or GST–HCV core fusion protein on glutathione–Sepharose beads were incubated with the *in vitro* translated. [^{35}S]methionine-labeled HCV core protein. The beads were boiled in Laemmli sample buffer, and the supernatants were analyzed by electrophoresis on 15% polyacrylamide gel containing SDS. Probe: *in vitro* translated HCV core protein. The full-length HCV core protein is indicated by an arrow.

core protein did not interfere with its homotypic interaction *in vitro*.

We have further studied the homotypic interaction between HCV core proteins by a Far-Western protein blotting assay. In this assay, *E. coli* lysate containing the recombinant full-length HCV core protein was separated by SDS–PAGE and transferred to nitrocellulose membrane. Proteins on the membrane were denatured, renatured, and incubated with ^{35}S-labeled, *in vitro* translated HCV core protein. Hepatitis delta antigen (HDAg) of hepatitis delta virus (HDV) was used as a control. Figure 3 shows that *in vitro* translated HCV core protein bound to the recombinant HCV core protein, but not HDAg. Since both HDAg and HCV core protein are highly basic nucleocapsid proteins (Chang *et al.*, 1988; Bukh *et al.*, 1994), and their sizes and biochemical properties are similar, this result suggests that the homotypic interaction of HCV core protein requires a specific sequence, which cannot be substituted by basic amino acid residues of other proteins. These results combined indicate that HCV core protein can undergo specific homotypic interaction directly.

Multimerization of HCV core protein in mammalian cells

To demonstrate the biological significance of the homotypic interaction of HCV core protein, we determined whether this protein exists as multimers when it is expressed in mammalian cells. We transfected COS 7 cells with expression plasmids which contained the full-length or truncated core protein-coding region under the CMV immediate-early promoter (Fig. 1). The proteins in the cellular lysates were cross-linked with glutaraldehyde, and the HCV core protein was detected by immunoblotting. The results showed that the glutaraldehyde treatment caused the appearance of a dimeric-sized (40-kDa) HCV core protein (Fig. 4A). As the treatment time was increased, an additional band of approximately 60 kDa appeared, which may represent core protein trimers. Correspondingly, the amounts of monomer protein (20 kDa) decreased as the length of treatment was increased. During treatments lasting more than 10 min, the amounts of 40- and 60-kDa proteins did not increase any further. However, there was a continuous increase in the amount of the proteins which did not enter the gel and a corresponding reduction of the monomer protein, suggesting the formation of bigger protein complexes. Since lysates contained numerous cellular proteins in addition to the HCV core protein, the detection of the discrete species of high-molecular-weight forms of the core proteins after cross-linking is indicative of the formation of specific complexes involving distinct protein species. The 40-kDa protein likely represents the dimeric form of HCV core protein (20 kDa), while the 60-kDa protein corresponds to the trimer form. The protein complex at the top of the gel may represent multimer complexes. These results thus indicate that HCV core protein forms multimers *in vivo*. The dimers and trimers detected probably represent the intermediates of multimeric complex formation. When truncated HCV core proteins were expressed, similar results were obtained (Figs. 4B and 4C). Without glutaraldehyde cross-linking, HCV core proteins a.a. 1–153 and 1–115 existed as proteins of 18 and 16 kDa, respectively. After glutaraldehyde cross-linking, protein bands of dimeric size, and possibly also trimeric size, were detected. Interestingly, the N-terminal hydrophilic portion (a.a. 1–115) of the core protein formed mostly multimeric forms; very little dimeric or trimeric form was detected

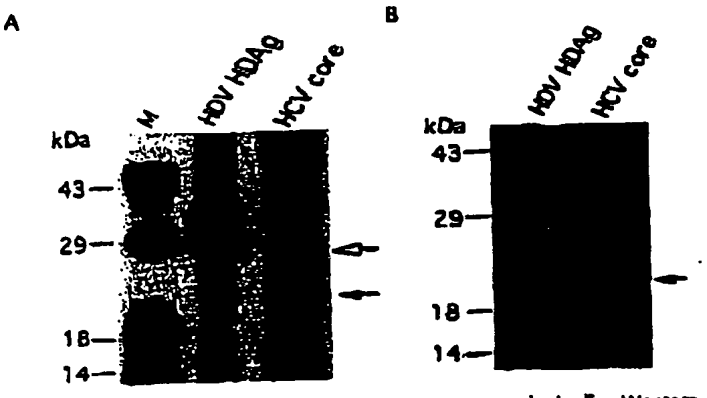

FIG. 3. Homotypic interaction of HCV core protein in Far-Western protein blotting assay. *E. coli*-expressed HCV core protein and baculovirus-expressed hepatitis delta antigen were separated by SDS–PAGE on a 15% polyacrylamide gel, and stained with Coomassie brilliant blue (A) or electrotransferred to a nitrocellulose membrane (B). The proteins on the membrane were denatured and renatured and incubated with *in vitro* translated, [^{35}S]methionine-labeled HCV core protein. Protein binding was detected by autoradiography (B). M: molecular size marker (in kilodaltons). HCV core is indicated by a solid arrow, and HDV HDAg is indicated by an open arrow.

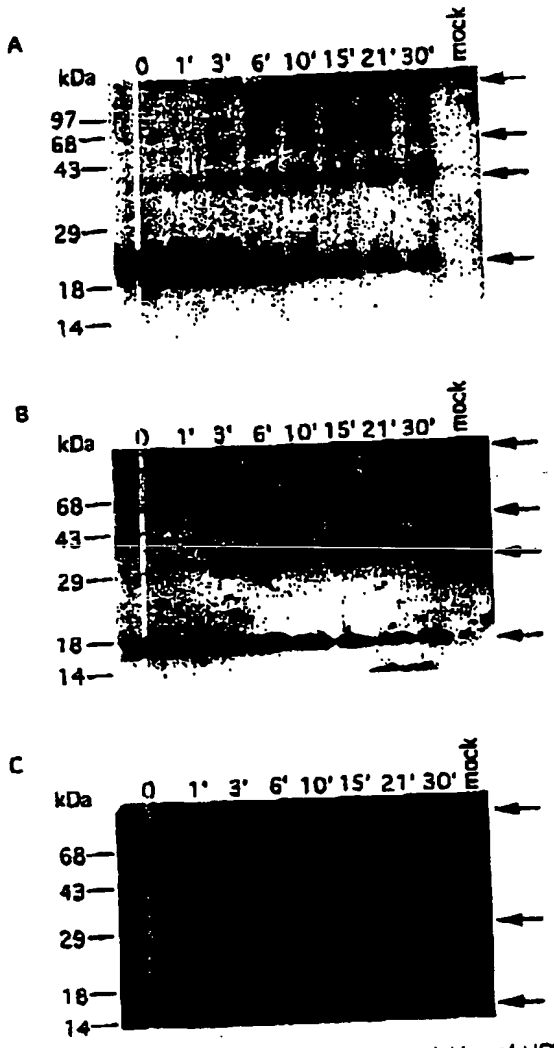

FIG. 4. Kinetic studies of glutaraldehyde cross-linking of HCV core protein expressed in COS 7 cells. COS 7 cells were transfected with expression plasmids pCMV/core(1-191) (A), pCMV/core(1-153) (B), or pCMV/core(1-115) (C). Cells were harvested 48 hr after transfection. Cell lysates were treated with 0.01% glutaraldehyde for 1', 3', 6', 10', 15', 21', or 30' at room temperature. HCV core protein was separated on SDS-containing 12.5% polyacrylamide gel and detected by immunoblotting using a polyclonal antibody against HCV core. 0: without cross-linking. 1', 3', 6', 10', 15', 21', and 30' indicate lengths (in min) of glutaraldehyde treatment. Mock: transfected with vector plasmid. Arrows indicate monomer, dimer, and trimer forms of HCV core protein, respectively, from the bottom. Top arrow indicates multimer which did not enter the gels.

(Fig. 4C). These findings indicate that the N-terminal hydrophilic region of the core protein is responsible for its multimerization. This result is consistent with the observation using the yeast two-hybrid system, in which HCV core protein interacted with itself through the middle region of the N-terminal hydrophilic domain.

Subcellular localization of the multimeric forms of HCV core protein

Since most of the HCV core protein has been shown to be associated with the endoplasmic reticulum (ER) (Santolini *et al.*, 1994; Lanford *et al.*, 1993), we wanted to examine whether multimerization of the protein *in vivo* requires the presence of the membrane. We separated the cellular lysates by a membrane flotation method (Sanderson *et al.*, 1993) into membrane and soluble fractions. HCV core protein in each fraction was detected by immunoblotting. Figure 5A shows the results with the full-length core protein. A majority of the core protein was recovered in the membrane fraction (Fig. 5A, lane 1), while only a small amount of the protein was recovered in the membrane-free form (fractions 4, 5, and 6). Interestingly, some of the core protein associated with the membrane already existed as dimeric and multimeric forms, even without glutaraldehyde cross-linking (Fig. 5A, lane 1). After glutaraldehyde treatment, the intensity of the dimeric (40 kDa) and multimeric forms increased (Fig. 5B). The dimeric and multimeric species were seen with both the membrane-associated and the membrane-free fractions, although the latter represented only a small fraction of the core protein. In contrast, most of the truncated forms (a.a. 1–153 and 1–115) of the core proteins were membrane-free (Figs. 5C and 5E, lanes 4, 5, and 6). After glutaraldehyde treatments, the dimer and multimer forms were detected in both the membrane-associated and the membrane-free fractions (Figs. 5D and 5F). In the case of a.a. 1–115 protein, most of the cross-linked proteins were in multimeric form (Fig. 5F), rather than the dimer form, consistent with the previous finding (Fig. 4). These results indicate that the multimerization of the core protein occurred in both membrane-associated and membrane-free forms in mammalian cells.

DISCUSSION

The data presented in this report demonstrated the homotypic interaction of HCV core protein *in vitro* and *in vivo*. Since the primary function of viral core proteins usually is to provide a protective shell for the viral genome inside, the ability of core proteins to multimerize to form a nucleocapsid would be an indispensable property of this structural protein. Indeed, in many viruses, nucleocapsid proteins have been shown to dimerize and multimerize (Luban *et al.*, 1992; Franke *et al.*, 1994; Beames and Lanford, 1993; Birnbaum and Nassal, 1990; Zhou and Standring, 1992; Chang *et al.*, 1994). HCV core protein would be expected to have similar properties. However, the structure of HCV nucleocapsid is still poorly understood. It is not known how the HCV core proteins assemble into a nucleocapsid structure. The finding in this report that this protein forms multimers *in vitro* and *in vivo* provides a structural basis for the viral nucleocapsid formation of HCV.

Our data showed that the hydrophilic domain (a.a. 1–116) is mainly responsible for the homotypic interaction of HCV core protein *in vitro* and *in vivo*. This conclusion was reached from the studies in the yeast two-hybrid

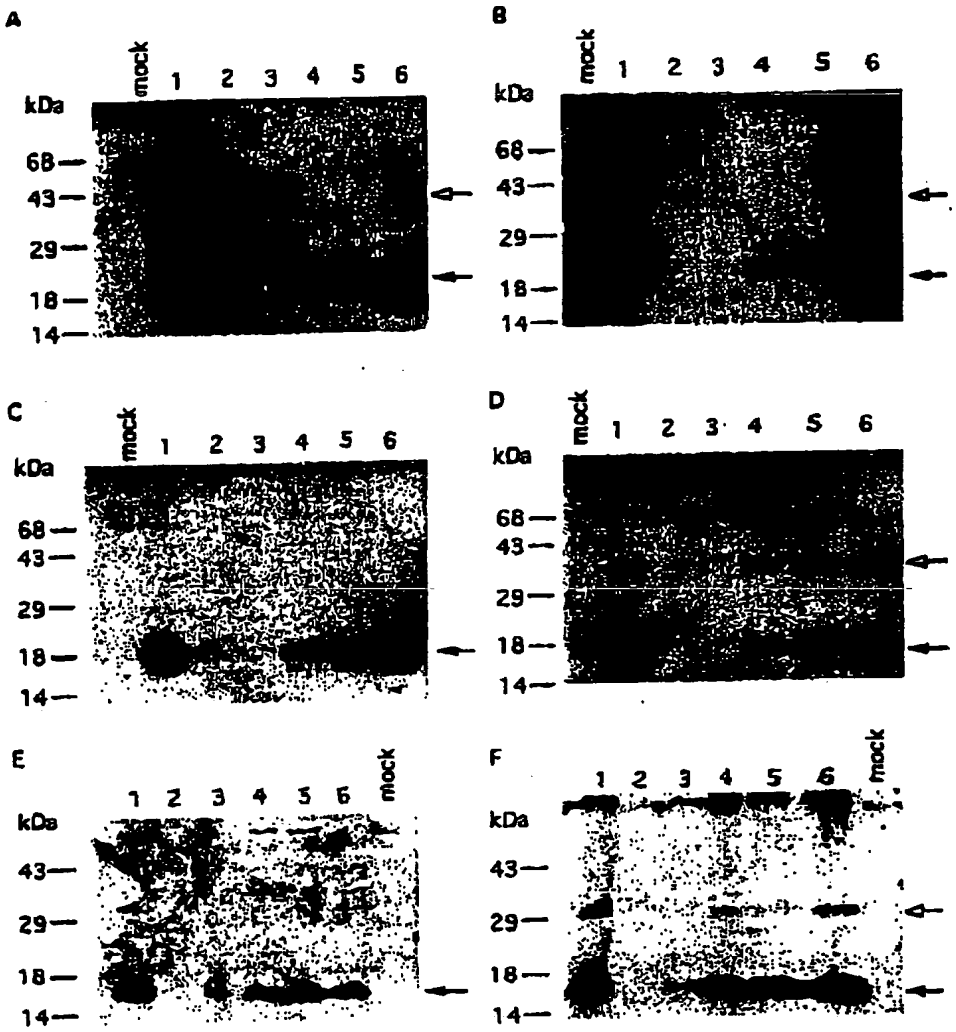

FIG. 5. Membrane flotation analysis of HCV core proteins expressed in COS 7 cells. COS 7 cells were transfected with expression plasmids, pCMV/core(1-191) (A and B), pCMV/core(1-153) (C and D), or pCMV/core(1-115) (E and F), and cells were harvested 48 hr after transfection. Cell lysates were fractionated by equilibrium sucrose gradient centrifugation and fractions were collected from the top of the gradient. The pelleted proteins were resuspended in buffer and designated as fraction 6. The remaining sucrose fractions were numbered from 1 to 5 in the order of top to bottom (light to heavy) fractions. Recovered proteins were analyzed either directly without glutaraldehyde treatment (A, C, and E) or after treatment with 0.01% glutaraldehyde for 15 min (B, D, and F). Solid arrows indicate monomer and open arrows indicate dimer. Mock: transfected with vector plasmid.

system, *in vitro* protein–protein binding assays, and the protein cross-linking experiments in mammalian cells. This hydrophilic domain is rich in basic amino acid residues and is very conserved among different HCV isolates (Bukh *et al.*, 1994). The protein–protein interaction of HCV core protein appears to require a specific amino acid sequence, inasmuch as HDAg, which has biochemical properties and sizes similar to those of HCV core protein, did not interact with the latter. The precise amino acid residues involved in the homotypic interactions remain to be determined. The yeast two-hybrid system tentatively mapped the homotypic interacting domain at a.a. 36–91, which overlaps the ribosome-binding and RNA-binding domains (a.a. 1–75) (Santolini *et al.*, 1994), but is outside the domain responsible for the suppression of HBV gene expression (a.a. 101–122) (Shih *et al.*, 1993). However, a.a. 36–91 alone appear to be insufficient for efficient protein binding; the neighboring sequences likely contributed to the homotypic interaction. It is not clear whether the partial overlapping of the protein-interacting and RNA-binding domains of HCV core protein have any functional significance. In this regard, it has been shown that the multimerization domain of NS1 protein of influenza virus overlaps its RNA-binding domain (Nemeroff *et al.*, 1995) and that the multimerization of Rev protein of human immunodeficiency virus 1 also involves its RNA-binding domain and adjacent sequences (Olsen *et al.*, 1990; Zapp *et al.*, 1991). One potential out-

core of this overlap of functional domains is that multimerization of core protein may alter the conformation of RNA-binding domain, or vice versa, to facilitate RNA–protein interactions in virus assembly. It would be interesting to know whether homotypic interaction is required for other functions of the core protein. The homotypic interacting domain also includes a purported phosphorylation site, Ser-53, but not Ser-99 or Ser-116 (Shih *et al.*, 1995). It is not clear whether phosphorylation can affect the homotypic interaction of the core protein.

The role of the hydrophobic domain (a.a. 116–191) in the homotypic interaction of HCV core protein remains to be investigated. We found that the presence of this domain interfered with protein interaction in the yeast two-hybrid system. However, this result was probably an artifact of this experimental system, since the presence of the hydrophobic sequence may interfere with the nuclear transport of the fusion protein in the yeast, thus invalidating this assay. It has been shown that the C-terminal hydrophobic region is responsible for the association of HCV core protein with ER membrane (Lanford *et al.*, 1993; Santolini *et al.*, 1994) and that the full-length core protein remained in the cytoplasm, whereas the truncated core protein, devoid of the C-terminal hydrophobic domain, was translocated into the nucleus (Suzuki *et al.*, 1995; Lo *et al.*, 1995). However, the full-length HCV core protein could undergo homotypic interaction *in vitro* (as determined by GST fusion protein binding assay and Far-Western protein blotting assay). Thus, it appears that the presence of the hydrophobic domain would not interfere with the core protein interaction. Nevertheless, the presence of the hydrophobic sequence may lower the efficiency of protein multimerization, as evidenced by our finding that the hydrophilic portion of HCV core protein (a.a. 1–115) tends to form bigger complexes than the proteins containing the hydrophobic domain in addition (Figs. 4 and 5). This result suggests that the truncated core proteins may be more easily multimerized and, therefore, preferentially incorporated into viral nucleocapsid. At the current time, the precise length and nature of the core protein species in the HCV nucleocapsic are not known.

HCV core protein is known to be produced by cleavage with host signal peptidase inside the ER at the N terminus of the E1 sequence of the polyprotein precursor; the mature core protein is then translocated into the cytoplasm (Santolini *et al.*, 1994; Selby *et al.*, 1993). The major ity of the core protein is still associated with the ER (Santolini *et al.*, 1994), and some may be translocated into the nucleus (Shih *et al.*, 1993; Lanford *et al.*, 1993; Lo *et al.*, 1995). Our data showed that both membrane-associated and membrane-free HCV core proteins formed multimers, suggesting that multimerization of the core protein may occur while the protein is still associated with the ER membrane. This multimerization step may represent the first step of virion assembly.

We could resolve only two of the discrete oligomers, including dimer and trimer, under our experimental conditions; the large aggregate of HCV core protein which did not enter gels may represent larger multimer complexes. The multimeric form was the major cross-linked protein species derived from the truncated HCV core protein that contains only the hydrophilic domain (Figs. 4 and 5). It is not clear what the size of this large complex is. Nevertheless, these large complexes are probably the precursor to nucleocapsid formation. Further studies on the properties of this multimer protein species will likely yield insights into the mechanism of virus assembly.

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